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Growth, sucrose synthase, and invertase activities of developing *Phaseolus vulgaris* L. fruits

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ABSTRACT

Activities of the sucrose-cleaving enzymes, acid and neutral invertase and sucrose synthase, were measured in pods and seeds of developing snap bean (*Phaseolus vulgaris* L.) fruits, and compared with ^{14}C-import, elongation and dry weight accumulation. During the first 10 d post-anthesis, pods elongated rapidly with pod dry weight increase lagging behind by several days. The temporal patterns of acid invertase activity and import coincided closely during the first part of pod development, consonant with a central role for this enzyme in converting imported sucrose during pod elongation and early dry weight accumulation. Later, sucrose synthase became the predominant enzyme of dry weight accumulation and was possibly associated with the development of phloem in pod walls. Sucrose synthase activity in seeds showed two peaks, corresponding to two phases of rapid import and dry weight accumulation; hence, sucrose synthase was associated with seed sink growth. Acid invertase activities in seeds were low and did not show a noticeable relationship with import or growth. All neutral invertase activities, during pod and seed development, were too low for it to have a dominant role in sucrose cleavage. Changes in activities of certain sucrose-cleaving enzymes appear to be correlated with certain sink functions, including import, storage of reserves, and biosynthetic activities. The data supports the association of specific sucrose-cleaving enzymes with the specific processes that occur in the developing pods and seeds of snap bean fruits; for example, acid invertase with pod elongation and sucrose synthase with fruit dry matter accumulation.

Key-words: Phaseolus vulgaris; bush bean; bean seed; ^{14}C-import; growth rate coefficient; invertase; sink metabolism; sink strength; sucrose metabolism; sucrose synthase.

INTRODUCTION

Sucrose is the major carbohydrate imported by many plant sink tissues. Since sucrose is not a direct substrate for most of the processes involved in growth, development and storage in most sinks, conversion of sucrose to hexoses often is the primary starting point for sink metabolism (Sung *et al.* 1988; Sung, Loboda & Black 1990; Xu *et al.* 1989b). Huber & Akazawa (1986) presented the concept of two distinct pathways for sucrose degradation, one mediated by sucrose synthase (SS) and the other by invertase, but the functional significance of the multiple pathways was not clear. Sung *et al.* (1988) also described parallel pathways each incorporating a different enzyme for the conversion of sucrose to hexose phosphates in higher plants and proposed that these alternative pathways have roles during development and as plants respond to environmental changes. It is conceivable that different sucrose-cleaving enzymes and related pathways may be used in connection with different biosynthetic or storage processes. For instance, Doehlert (1990) concluded that different parts of developing maize kernels differ in their enzyme composition, reflecting the differences in their storage product accumulation. Imported sucrose supplies carbon for a host of processes in sink tissues, and consequently, the pathway of conversion present may depend on the processes occurring in the sink at that time. In many actively growing and storing sink tissues, SS activity is quite high (Ross & Davies 1992; Sun *et al.* 1992; Sung *et al.* 1989a, 1990; Sung, Xu & Black 1989b; Xu *et al.* 1989a). In others, particularly in those undergoing expansion (Morris 1982; Morris & Arthur 1984), acid invertase (AI) activity is associated with high sink activity. However, in some sucrose storage organs, AI activity decreases with increasing sucrose accumulation (Hubbard, Huber & Pharr 1989; Miron & Schaffer 1991).

In attempting to identify biochemical indicators of sink strength, it was proposed that the activity of SS can serve as an indicator of active sink growth (Sun *et al.* 1992; Sung *et al.* 1989b). That hypothesis was derived from studying plant sinks such as developing tomato fruits, bulking potato tubers, and developing lima bean seeds (Sun *et al.* 1992; Sung *et al.* 1989b; Xu *et al.* 1989a). Particularly with lima bean seeds and tomato fruits, the activity of SS was strongly related to growth. These sinks exhibit classical single phase logarithmic growth curves. But it also has been known that certain plant sinks (e.g. *Phaseolus vulgaris* seeds) show biphasic growth curves (Carr & Skene 1961; Geiger, Shieh & Saluke 1989) and many fleshy drupaceous fruits even show triphasic growth curves

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(Coombe 1976). Therefore, it was reasoned that plant sinks with two or three growth phases could be used to test the hypothesis that the total SS activity per organ is an indicator of sink strength of that organ. If SS is the predominant enzyme for cleaving the imported sucrose used to support growth of a particular sink, the developmental time course for total SS activity should follow the same pattern as the growth rate of this sink.

Here data are presented on snap beans, contrasting pods, which show a single phase growth, with seeds which show biphasic growth. The complete snap bean fruit is an attractive test system because the large pod initially competes with the seeds and marked partitioning changes occur within the fruit during development. The study was undertaken to investigate possible relationships between the sucrose-cleaving enzymes AI, SS and neutral invertase (NI), and the sink processes of carbon import, growth in size and accumulation of dry weight during bean fruit development. Temporal (DPA) and spatial (pod versus seed) patterns were studied in *Phaseolus vulgaris* fruits, whose development also has been characterized in other types of studies (Geiger *et al.* 1989; Loewenberger 1955; Oliker, Poljakoff-Mayber & Mayer 1978; Sage & Webster 1990; Walbot, Clutter & Sussex 1972). The activities of AI and SS differed between pods and seeds and also changed during development of each structure. These enzyme activity patterns appear to be related to the temporal appearance of various sink processes in developing pod and seeds.

MATERIALS AND METHODS

Plant material

Snap bean, *Phaseolus vulgaris* L. cv Black Valentine, seeds were sown in 4·0 dm^3 plastic pots and thinned to three plants per pot. Plant growth conditions in Dayton were described previously (Geiger *et al.* 1989). In Athens, similar growth and cultural conditions were employed except the plants were grown in a green-house. Each day individual anthesizing flowers were tagged near 1100 h. Development was determined at intervals of days post anthesis (DPA) to maturity, a 32–35-d period, by measuring dry weight, length, and various features of the pod and seeds. Since each fruit was tagged on the day of anthesis, fruits that showed slower than average growth rate also were identified as slow growing pods and sampled for separate analysis. On the average, there were five seeds within a snap bean fruit and we mostly assayed individual seeds and pod.

Carbon import and analysis of growth rate

To reduce the possible diurnal effects on measurements, fruits were collected daily near 1100 h from anthesis to 35 DPA and the dry weight of pods and seeds were measured. The daily rates of dry weight increase were calculated from smoothed curves for dry weight accumulated per pod or seed (Geiger & Shieh 1988; Geiger *et al.* 1989). To determine import rate, $^{14}CO_2$ of constant known specific radioactivity and concentration was supplied to the entire shoot throughout a 14-h light period (Geiger & Shieh 1988; Geiger *et al.* 1989). Fruits were removed at the end of the night period, 24 h after the start of labelling, seeds and pods were separated, and each piece was divided into two halves. The half pieces to be used for enzyme assay were rapidly frozen and stored individually in liquid N_2 until analysis. Corresponding half-pieces of pods or seeds were frozen, dried and powdered. The ^{14}C-content was determined by oxidizing aliquots of the powder from each piece (Geiger & Shieh 1988).

The ^{14}C which had accumulated in a pod or seed was used to estimate the total imported carbon minus respiration in each organ during the 24-h period. A growth rate coefficient (GRC) was calculated for each pod or seed from the following data: the specific radioactivity of the $^{14}CO_2$, the ^{14}C-content of an aliquot of the organ, its total dry weight, and the proportion of dry weight as carbon (Geiger & Shieh 1988). The GRC, which is a measure of grams carbon gained daily per gram dry weight of that organ, is an estimate of sink activity. Sink strength generally is considered to be the product of sink activity and sink size (Warren Wilson 1972). We estimated sink activity by GRC, sink size by dry weight, and sink strength by the total dry weight imported per day. The amount of carbon respired by the organ was not measured in the calculation of import rate. Hence, the import rate we calculate is an integrated value for the net increase in recently assimilated carbon that was imported during a 24-h period. We estimate respiration to be 10% in mature seeds and up to 40% in the earlier embryos. The daily dry weight increase was generally 10–20% larger than the estimate of imported carbon but the patterns were in good agreement.

Enzyme assays

Enzyme analyses were carried out on halves of pods and seeds. Early in development, when a single pod or seed was too small to analyse, several pods or seeds were combined. Data from enzyme assays conducted with freshly harvested tissues were comparable to those from liquid N_2 frozen tissues. One gram of bean pod walls or 0·25 g of bean seeds were powdered in liquid N_2 with a mortar and pestle. Extraction buffer, at a 5:1 (v:w) ratio of buffer:tissue, and 1% (w/w) insoluble PVP, 1% (w/w) Dowex-1, 0·02 mol m^{-3} PMSF, and sand were then added to homogenize tissues. The extraction buffer contained 200 mol m^{-3} Hepes/NaOH (pH 7·5), 3 mol m^{-3} magnesium acetate, 5 mol m^{-3} dithiothreitol (DTT), 1% (v/v) glycerol, and 1% (v/v) Triton-X 100. The homogenate was filtered with one layer of Miracloth and centrifuged at 34 000 ***g*** for 15 min. The supernatant was desalted with a Sephadex G-25 column. Protein was eluted with 25 mol m^{-3} Hepes/NaOH (pH 7·5) containing 3 mol m^{-3} magnesium acetate, 5 mol m^{-3} DTT, and 10% (v/v) glycerol.

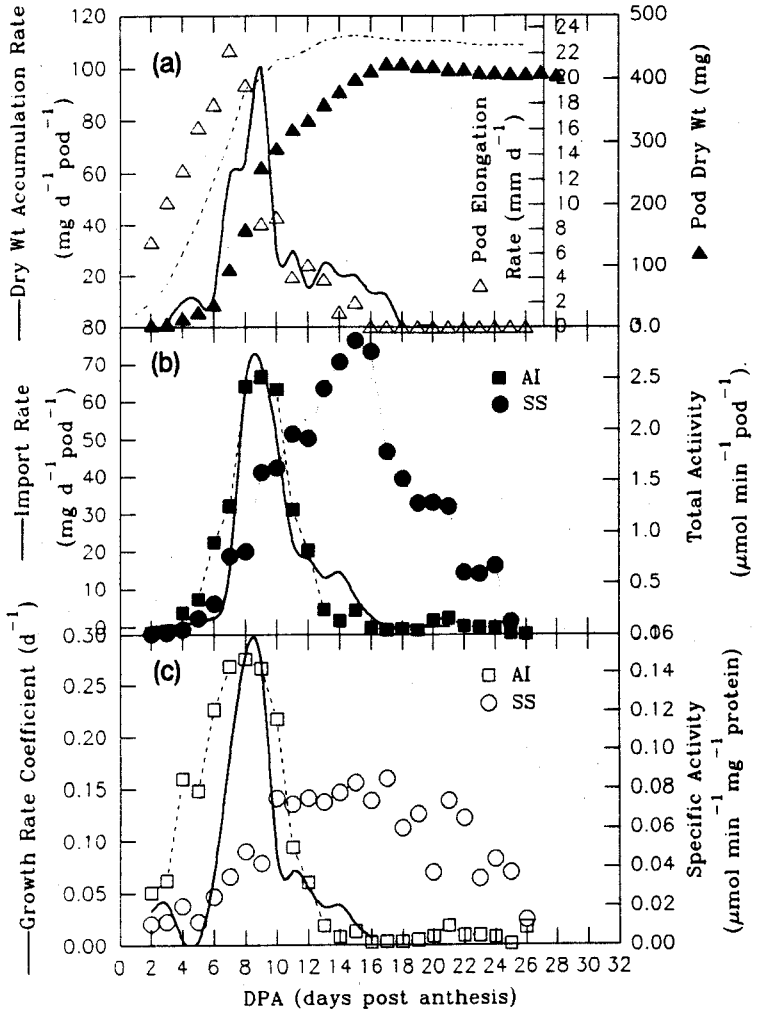

Figure 1. Time course of snap bean pod development: (a) pod dry weight (▲), dry weight accumulation rate (solid line) and pod elongation rate (△). The dashed line indicates pod length with a maximum of 12 cm; these units are not given on an ordinate: (b) import rate (solid line) and total soluble acid invertase (■) and total sucrose synthase activity (●); (c) growth rate coefficient (solid line) and specific soluble acid invertase (□) and specific sucrose synthase activity (○).

Enzymes were assayed immediately after extraction. All activities were proportional to the amount of extract and to time. SS, AI and NI were assayed following the procedures of Xu *et al.* (1989a). SS was assayed by measuring the continuous change in OD at 340 nm at 25 °C on a Beckman DU-7 spectrophotometer using 100 mol m^{-3} sucrose, 0·5 mol m^{-3} UDP, and 1 mol m^{-3} PPi as substrates and phosphoglucomutase (1 U) and *Leuconostoc* Glu 6-P dehydrogenase (1 U) as coupling enzymes. Whenever there was less than 1 U of UDP-glucopyrophosphorylase present in the extract, 1 U of potato tuber UDP-glucopyrophosphorylase was added. The validation of stoichiometries and the requirements of this SS assay are available (Xu *et al.* 1989b). AI and NI were assayed with 25 mol m^{-3} sucrose at pH 5·0 and 100 mol m^{-3} sucrose at pH 7·0, respectively. Generally, after a 15 min incubation at 25 °C, the reaction was stopped by boiling for 7 min. The AI reaction mixtures were neutralized before boiling. The glucose formed then was measured using hexokinase (1 U) and *Leuconostoc* Glu 6-P dehydrogenase (1 U). Extract protein contents were determined using the Bradford procedure with BSA as the standard protein (Bradford 1976).

RESULTS

Pod development

During fruit development in *Phaseolus vulgaris*, pods develop to a substantial extent before rapid seed development begins (Geiger *et al.* 1989; Loewenberger 1955; Oliker *et al.* 1978; Walbot *et al.* 1972). In the present study, rapid pod elongation began nearly immediately after anthesis, several days before dry weight began to increase markedly (Fig. 1a). Final pod length was attained by about 10 DPA. Dry weight increased rapidly during the period of 6–9 DPA and then more slowly until about 17 DPA, after which the pod lost dry weight gradually.

Transient increases in the activities of AI and SS

occurred during pod development. Starting 3–4 d after anthesis, AI activity (Fig. 1b) increased rapidly to a peak at 9 DPA, the time of the maximum rate of dry weight accumulation, and then decreased to near zero by 14 DPA. Levels of both total and specific activity of pod AI coincided closely with the rate of total pod dry wt accumulation and GRC values, respectively (Fig. 1b, c). The maximum level of AI activity, which was attained at 9 DPA, was approximately 2·5 μmol glucose imported min^{-1} per pod. This level of AI activity was approximately ten times the 70 mg dry weight d^{-1} or 0·27 μmol glucose min^{-1} imported per pod at this stage of development. The close correspondence between the time courses of AI activity and import is consonant with a central role for AI in converting imported sucrose to pod dry weight during pod development. The activity of NI in pods was low and the developmental time course of this activity did not show an evident relationship with import rate (data not shown).

SS activity increased steadily during the period of rapid increase in pod dry weight (Fig. 1b), reached a maximum by 15 DPA and decreased to near zero by 26 DPA. During the most rapid import and dry weight accumulation period (7–10 DPA), SS activity was much lower than AI activity. However, between 12 and 17 DPA when pod dry weight accumulation was continuing at a lower rate, SS became the predominant sucrose cleaving enzyme in the pod (Fig. 1b).

It is common to observe slower-growing fruits, and in earlier studies, it was observed that import rate was particularly low in certain developing fruits (unpublished data). Measurements of pod length and weight confirmed that the growth of some fruits paused or continued at a slow rate during development (Table 1). Usually only one to three seeds developed in these slow-growing fruits. The specific activities of AI and SS were reduced correspondingly in pods of such fruits consistent with their having an important role in fruit development (Table 1).

Table 1. Acid invertase and sucrose synthase specific activities in slow-growing snap bean pods compared to controls. Number in the parenthesis following a given value indicates per cent reduction compared to the control

DPA	Fresh weight (mg)	Length (mm)	Specific activity AI (nmol mg^{-1})	Specific activity SS (protein min^{-1})
6	188 (78)	37 (48)	51 (58)	9 (76)
8	164 (95)	33 (71)	7 (95)	7 (89)
9	1210 (70)	72 (39)	79 (35)	18 (73)
10	1345 (75)	75 (43)	3 (97)	8 (89)
11	790 (85)	68 (47)	5 (90)	6 (91)
12	1226 (77)	87 (31)	15 (48)	6 (91)
13	2075 (63)	104 (20)	5 (37)	8 (88)
15	2780 (64)	95 (37)	13 (0)	30 (60)
16	3364 (57)	102 (26)	3 (0)	38 (45)
17	2604 (66)	88 (41)	5 (0)	19 (68)

Seed development

Seed dry weight increases in two stages (Carr & Skene 1961; Geiger *et al.* 1989), a phenomenon termed diauxic growth (Carr & Skene 1961). Seed size begins to increase markedly several days prior to the rapid increase in dry weight and reaches a maximum at about 20 DPA (Geiger *et al.* 1989). After this time, dry weight continues to increase but without a marked increase in seed size. The initial rapid increase in dry weight occurred during 12–17 DPA and the second one during 20–28 DPA (Fig. 2a).

There were two phases of transient changes in SS activity per seed and these corresponded to the two phases of import and growth (Fig. 2a, b). Activity per seed initially increased rapidly to a peak at 16 DPA, decreased, and then attained a second peak at 24 DPA, times of maximum rates of import and dry weight accumulation. SS activity reached a top value of approximately 1000 nmol glucose min^{-1} per seed at 24 DPA while import rate was 17 mg dry weight d^{-1} or 66 nmol glucose min^{-1}. The measured rate of enzyme activity was 15 times the rate needed to keep pace with the conversion of sucrose imported by a seed at this stage of development. The activities of AI and NI in seeds were less than 9 nmol glucose min^{-1} mg $protein^{-1}$, too low to process the imported sucrose, and the developmental time course of their activities did not show an evident relationship with import rate (data not shown).

Very early in seed development, the seed GRC was high (Fig. 2c), simultaneously with the peak pod GRC (Fig. 1c). No doubt the pod and seeds were in competition then for imported sucrose, but the seeds were only able to dominate later as its dry matter accumulated more rapidly. Because storage is an important part of seed development, dry weight accumulates with little or no increase in metabolically active tissues, and so GRC decreased markedly with time (Fig. 2c).

A comparison of the pod versus seeds in a complete snap bean fruit

Time courses for import and enzyme activity for individual pods and seeds were combined to represent a typical fruit consisting of a pod and five seeds (Fig. 3). In this way, the relative import rate into pod and seeds are put into the perspective of a single importing unit. The import rate, measured by import of labelled carbon, exhibited three distinct peaks (Fig. 3a). The first corresponded to a large AI peak in pods (Fig. 1b) and the other two peaks corresponded to two large SS peaks in seeds (Fig. 2b). The GRC for the whole fruit also exhibits three distinct peaks consonant with carbon import rate along with AI in pods plus SS in the seeds (Fig. 3a, b).

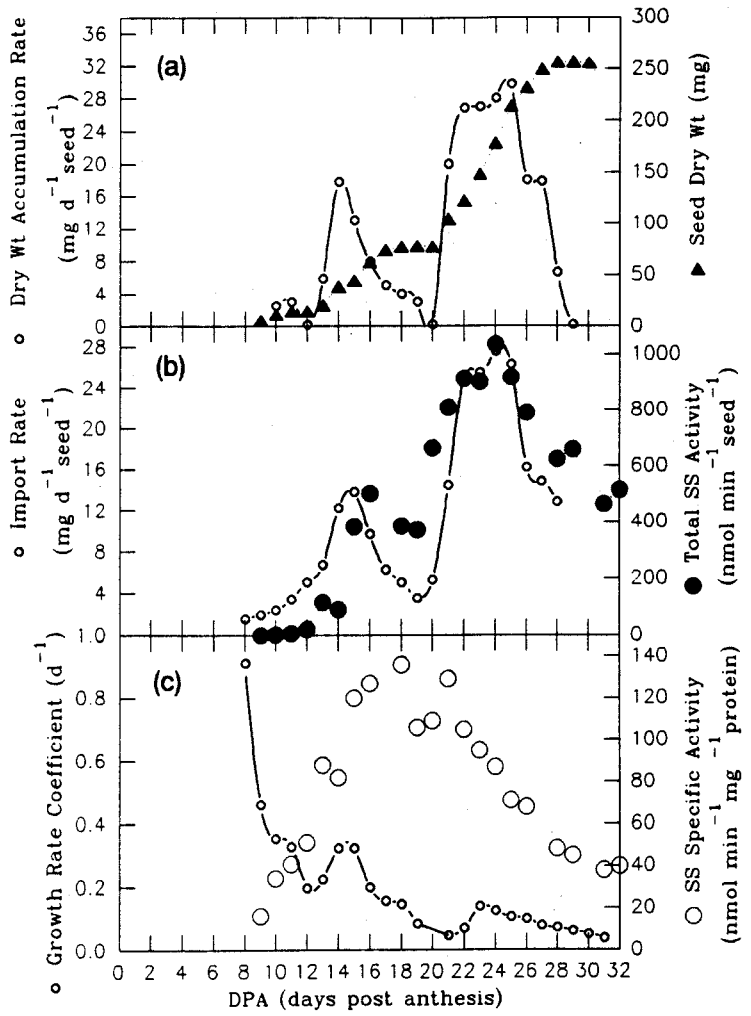

Figure 2. Time course of snap bean seed development: (a) seed dry weight (▲) and dry weight accumulation rate (-○-); (b) import rate (-○-) and total sucrose synthase activity(●); (c) growth rate coefficient (-○-) and specific sucrose synthase activity (○).

Protein extractability and other enzyme activities

In addition to these three sucrose cleavage enzymes, the activities of UDP-glucopyrophosphorylase, pyrophosphate (PPi) and ATP-dependent phosphofructo-kinase in developing pods and seeds were measured (these data are not presented). The PPi-PFK activity showed similar patterns as those of SS in both pods and seeds. Both ATP-PFK and UDP-glucopyrophosphorylase activities were practically constant throughout development in each tissue. The facts that some enzyme activities (e.g. ATP-PFK) did not decrease with development and that total extractable soluble protein contents varied less than two-fold throughout development indicated that enzyme extractability did not change detectably as these tissues developed. Also in preliminary work, studies were conducted on the coextraction of protein and enzymes by combining bean fruit tissues with actively bulking potato tubers. Greater than 95% recoveries were obtained (data not shown).

DISCUSSION

Relation of enzyme activities to rates of import and metabolism

The measured activities of these enzymes (SS and AI, Figs 1 & 2), which were assayed under near-optimum conditions, generally were sufficient to cleave sucrose at rates 10 to 15 times the observed net carbon import rate. A similar relationship between values for SS activity and import rates was observed in tomatoes (Sun *et al.* 1992). Presumably sucrose concentrations at the sites of cleavage in sink tissues were considerably lower than that present in the assay mixtures. Sucrose concentrations are likely to be reduced in sink tissues because short distance sucrose transport occurs by diffusion which is driven by lowering the sucrose concentration at the destination point (Thorne 1980, 1981). None of the measured NI activities were sufficient to process the amount of sucrose imported either by pods or seeds of snapbeans. However, a sucrose cleavage

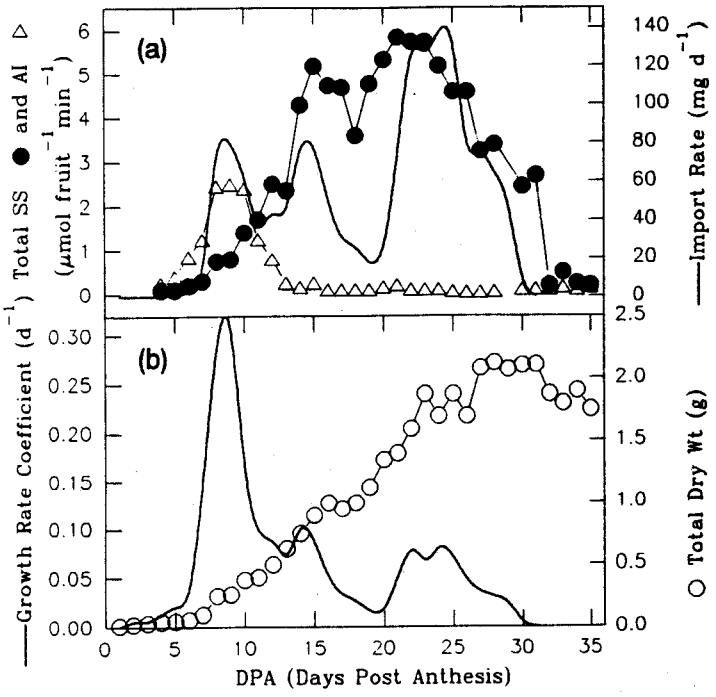

Figure 3. Time course of development of a typical snap bean fruit constructed by combining data for one pod plus five seeds: (a) import rate (solid line), total acid invertase (△) and total sucrose synthase activity (●); (b) growth rate coefficient (solid line) and total fruit dry weight (○).

role for NI has been proposed in the developing pea pod (Estruch & Beltran 1991).

The fact that the developmental patterns of total AI and SS activity per organ agreed with those of import into that organ indicates that these enzymes likely are involved in cleaving imported sucrose, and thus, in initiating hexose metabolism in support of current sink processes. However, the fact that the level and time course of enzyme activity correspond to various phases of development is not sufficient to establish that these enzymes are the principal point of regulation of import or metabolism rate. Presumably the associated biochemical processes for synthesizing structural and storage materials in pods and seeds also have a role in regulating both growth and import. The various processes associated with conversion and use of imported sucrose likely will be coordinated in the growing sinks. A fine-tuned regulatory mechanism appears to control the sequential growth and development of pod and seeds (Oliker *et al.* 1978).

Significance of particular enzymes in specific sink processes

The activity of sucrose-cleaving enzymes not only differs between pods and seeds but also temporally for a given structure. These differences likely reflect the nature of sink processes currently taking place in a given organ (Warren Wilson 1972). Doehlert (1990) examined clusters of enzymes that are involved in a number of biosynthesis pathways in maize and found that the activity levels of a certain cluster of enzymes reflect the extent to which the particular biosynthetic activity is occurring in that tissue. For instance, in developing maize kernels, invertase in the embryo mediates the carbon supply for oil synthesis while SS mediates starch and cell wall synthesis. The temporal and spatial patterns of enzyme activities which we observed for AI and SS in the present study presumably are related to the specific sink processes occurring there. For example, in seeds the first SS peak occurs concurrent with cell division and seed coat protein biosynthesis while the second SS peak mediates the biosynthesis of storage proteins and starch (Geiger *et al.* 1989).

The critical time during which a *Phaseolus vulgaris* fruit either will continue to develop or abort is 4 DPA (Sage & Webster 1990) and this is the time when AI activity and pod dry weight begin to increase rapidly (Fig. 1a, b). The high AI activity observed during the first half of pod development in the present study appears to be involved in cleaving sucrose for metabolism in support of pod elongation and accumulation of reserve and structural materials. Later in development, pod SS increases to a high level at a time when the rate of dry weight accumulation is lower. These patterns likely can be accounted for by the series of processes which occur in the course of pod development.

Pods are considered to be fundamentally foliar in nature (Radford *et al.* 1974), importing assimilate early in their development and later exporting it to the developing seeds (Flinn & Pate 1970; Fader & Koller 1985; Oliker *et al.* 1978; Thorne 1980). As a consequence, the sequence of enzyme activity in pods is likely to resemble that which

occurs during leaf development. In leaves of *Phaseolus vulgaris*, invertase activity increases to a high level during the stage of rapid expansion and decreases markedly when leaves reach their final size (Morris & Arthur 1984; Pate *et al.* 1985). Likewise, the level of AI activity was high during pod elongation and then, as dry weight accumulation gradually decreased, AI activity decreased. SS became the predominate sucrose metabolizing enzyme at this stage when imported assimilates did not contribute much to pod dry weight. The increase in SS activity may be associated with the development of an extensive network of exporting phloem (Yang & Russell 1990), present in the walls of mature pods (Thorne 1981), which translocates assimilate, mainly from refixation of respiratory CO_2 (Flinn & Pate 1970; Crookston, O'Toole & Ozbun 1974; Oliker *et al.* 1978; Thorne 1980). Also, at this time, assimilates pass through the phloem of the pod on route to the developing seeds (Thorne 1980; Fader & Koller 1985).

A significant accumulation of seed dry weight begins about 8 DPA and is accompanied by rapid increases in SS activity. During the first period of dry weight increase in *Phaseolus vulgaris* seeds, from 8–17 DPA, stachyose begins to accumulate (Tanner, Seifarth & Kandler 1968), seed coat protein increases and seeds rapidly increase in volume (Fig. 4 in Geiger *et al.* 1989). The second period of rapid increase in seed dry weight begins about 20 DPA at the time of rapid starch accumulation. The increased SS activity at this time may serve to supply assimilate for starch synthesis (Opik 1968). Elevated SS activity was found to be associated with starch synthesis from imported assimilate in maize (Doehlert 1990). The pause in import, in dry weight accumulation, and the transient decrease in SS activity may result from specific processes either stopping or starting. For example, such a change in SS activity would be needed if sink processes occur in different cellular compartments in the seed, such as the cytosol and plastid, or in different structures, such as the embryo axis, cotyledon or seed coat. Another possible factor is that the pod and seed coat are maternal tissues while the embryo axis and cotyledons belong to the next generation. Or part of the SS activity may be associated with the extensive vascular network present in the seed coat (Offler & Patrick 1984).

CONCLUSIONS

Several lines of evidence were presented to show that the total enzyme activity for sucrose cleavage per organ was an indicator of growth rate and sink activity. For example, two peaks of SS activity corresponding to two peaks of dry weight accumulation in seeds; a lower level of activity in pods with a reduced dry weight accumulation; and low SS activities in mature pods and seeds. Evidence was also presented to show that AI was the major sucrose cleavage enzyme during early pod development. Later, during the period when they were no longer accumulating dry weight, SS was the dominant sucrose-cleaving activity in pods. Thus, within a same tissue, the growing snap bean pod, AI and SS were indicators of fruit growth, i.e. sink strength, at different times of development, presumably associated with changes in metabolism.

Changes in activities of sucrose-cleaving enzymes, which were observed during bean fruit development, indicate that particular enzymes assume greater or lesser importance in different tissues and at different times. The patterns of AI and SS activities were correlated with various sink functions, including import, storage of reserves, growth and various biosynthetic activities. The data are consistent with the association of specific sucrose-cleaving enzymes with various sink processes that occur in the developing pod and seeds of *Phaseolus vulgaris* fruits.

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